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# Pederin-Type Pathways of Uncultivated Bacterial Symbionts: Analysis of *O*-Methyltransferases and Generation of a Biosynthetic Hybrid

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The pederin family comprises a large number of complex polyketides that have been reported from remarkably disparate organisms.[1] While pederin (**1**) occurs in the rove beetle genera *Paederus* and *Paederidus* spp., the other compounds, such as onnamide A (**2**)[2] and the mycalamides A (**3**) and B (**4**),[3] were isolated from various marine sponges (Figure 1). From the total DNA of *Paederus fuscipes* beetles[4] and the sponge *Theonella swinhoei*,[5] we previously isolated architecturally highly similar polyketide synthase (PKS) genes whose structures closely correspond to the those of **1** and **2**, respectively.[4] Intriguingly, our data showed that in both animals, the genes belong to prokaryotic genomes,[4,5] suggesting that the polyketides are produced by symbiotic bacteria. To date, however, cultivation of the suspected producers has been unsuccessful, and no biochemical studies existed to prove the identity of the genes. In addition to these data, it was reported that pederin biosynthesis is strictly correlated with the presence of a *Pseudomonas* sp. bacterium,[6] but scenarios other than bacterial production, such as induced biosynthesis, cannot be excluded.

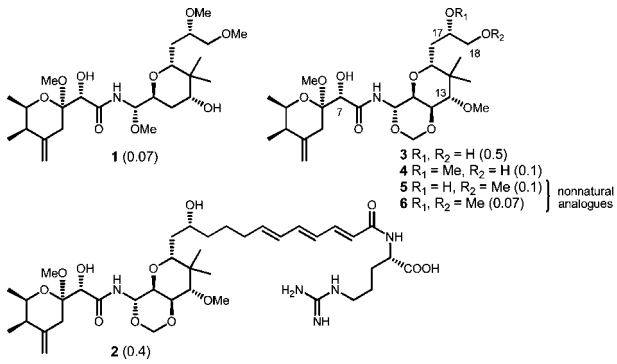


***Figure 1.*** Structures of pederin (**1**), onnamide A (**2**), and natural (**3**, **4**) and semisynthetic (**5**, **6**) mycalamides. Numbers in parentheses are $IC_{50}$ values (ng $cm^{-3}$) reported for P388 assays.[7]

Pederin-type compounds exhibit impressive cytotoxic activites down to the subnanomolar range.[1] It has been shown that the potency can be markedly increased by modifying the methylation pattern.[7] For the predominant compound **3** present in the sponge *Mycale hentscheli*, $IC_{50}$ values for a P388 cell-based assay increased 5-fold by single methylation at either C17 (**4**) or C18 (**5**), and the doubly methylated nonnatural analogue **6** was as potent as the most active compound, **1** (Figure 1). As chemical O-methylation of **3** had only resulted in compound mixtures,[7] we were interested in chemoenzymatic transformations that might provide access to regiospecifically methylated mycalamides. Importantly, this approach would also allow us to obtain a functional proof of the biosynthetic role of the genes and thus test the symbiont hypothesis.

The putative pederin biosynthetic gene cluster (*ped*) encodes three proteins with similarity to *O*-methyltransferases (MTs): PedA, PedE, and PedO.[4] To study their function, the corresponding genes were amplified by PCR and inserted into pMAL-c2x and pHis8-3 for expression as maltose-binding protein (MBP) fusions and octahistidyl-tagged enzymes, respectively. After introduction into *Escherichia coli* BL21(DE3) and optimization of expression conditions, we obtained soluble proteins from all of the constructs (Figure 2A). These were further purified by affinity column chromatography and stored at −80 °C. After incubation of each enzyme with the test substrate **3** and the methyl donor *S*-adenosylmethionine (SAM), analysis of the reaction mixture by electrospray ionization−time of flight mass spectrometry (ESI-TOF-MS) revealed that a new product with the deduced molecular formula $C_{24}H_{41}NO_{10}$ (*m*/*z* 540.274 measured, 540.278 calcd for $[M + Na]^+$) was formed for $His_8$- or MBP-tagged PedO (Figure 2B). This corresponds to a product carrying one more methylene unit than **3**, suggesting that PedO catalyzes a single methylation reaction. Assays with the other MTs contained only unconverted **3** even after prolonged incubation.

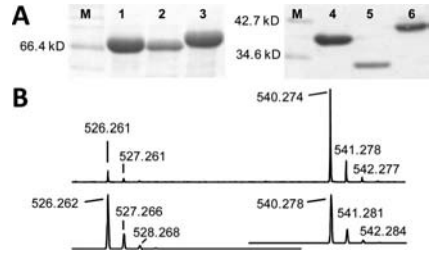


***Figure 2.*** (A) SDS-PAGE of the expressed *O*-MTs PedA (lanes 1 and 4), PedE (lanes 2 and 5), and PedO (lanes 3 and 6) as MBP fusion (lanes 1−3) and $His_8$-tagged (lanes 4−6) proteins. M denotes a protein marker. (B) High-resolution ESI-TOF mass spectrum of a reaction mixture with PedO−MBP and **3** (top) and calculated masses and isotopic patterns of $C_{23}H_{39}NO_{10}Na^+$ ($[\mathbf{3} + Na]^+$, bottom left) and $C_{24}H_{41}NO_{10}Na^+$ ($[\mathbf{3} + CH_2 + Na]^+$, bottom right).

Compound **3** contains three hydroxyl groups and an amide function that could potentially serve as nucleophilic methylation sites. To identify the correct position from the small amounts of available material (in total, 1.1 mg of **3** could be used for all of the enzyme assays), we performed $MS^2$ measurements on the quasi-molecular ion of **3** and the PedO product. The fragmentation pattern of $[\mathbf{3} + Na]^+$ (Figure 3A) is interpreted in terms of scissions around the central amide bond in addition to losses of methanol and formaldehyde (Figure 3B). The mass differences and intensities in the $MS^2$ spectrum of $[M + Na]^+$ of the PedO product are identical, but all of the ions are shifted by *m*/*z* 14 (Figure 3A). Since this includes the smallest detectable ions lacking the Western acyl amide

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moiety, methylation at the C7−OH or the nitrogen atom was ruled out, leaving either **4** or **5** as possible products.

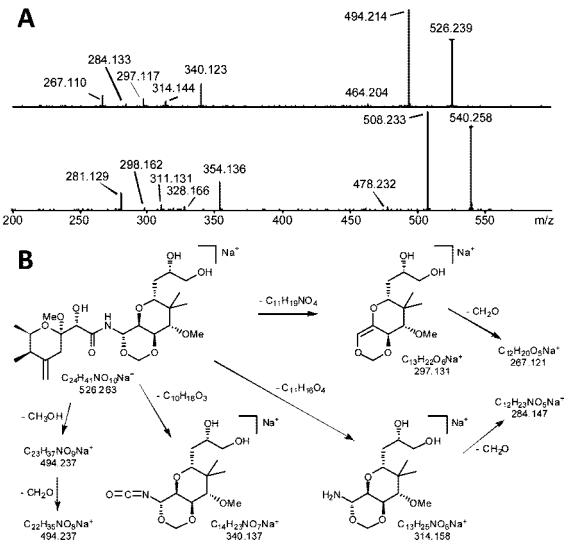


***Figure 3.*** $MS^2$ analysis of mycalamides. (A) Collision-induced dissociation $MS^2$ spectra of (top) the sodium adduct $[M + Na]^+$ of mycalamide A (**3**) and (bottom) the methylation product. (B) fragmentation scheme of the sodium adduct $[\mathbf{3} + Na]^+$. Exact mass differences confirmed the assigned elemental compositions.

A comparison of the methylated polyketide with authentic **4** isolated from *M. hentscheli* revealed identical HPLC retention times and the same $MS^2$ pattern for the two compounds. In addition, incubation of **4** with PedO did not result in the formation of new compounds, such as the terminally dimethylated analogue **6** that would arise from a methylation at the C18−OH group. These data appeared to suggest that PedO generated **4** from **3**. However, we could not exclude the possibility that PedO only attacks the C18 hydroxyl group if the neighboring position is unmethylated. In addition, LC−$MS^2$ analysis of semisynthetic **5** showed that it was indistinguishable from **4** regarding retention time and ion fragmentation. To unequivocally identify the methylation site, we therefore performed 1D and 2D NMR studies. Mycalamide A (30 $\mu$g) was incubated with MBP-fused PedO and SAM. After extraction and evaporation of the solvent, the product was dissolved in $CD_3OD$ and placed in a CapNMR probe, and $^1H$ NMR and heteronuclear single-quantum correlation (HSQC) spectra were obtained. The $^1H$ NMR spectrum of the product clearly showed five signals in the methoxyl region near $\delta_H$ 3.30, 3.35, and 3.55. The signals at $\delta_H$ 3.295 and 3.555 corresponded to the expected signals for 6-OMe and 13-OMe from unreacted **3** in the methylation mixture.[3] The three remaining −OMe signals [$\delta_H$ 3.299, 3.346 and 3.550] were from the methylation product, with the $\delta_H$ 3.299 and 3.550 signals assigned as 6-OMe and 13-OMe, respectively. If the 17-OH group had been methylated by PedO to give **4**, the expected 17-OMe signal would have been at $\delta_H$ 3.24.[3,7] However, the third signal was at $\delta_H$ 3.346, as observed for the semisynthetic 18-OMe mycalamide **5**.[7] The HSQC spectrum confirmed the 18-OMe assignment with the observation of a correlation at the expected values of $\delta_H$ 3.35/$\delta_C$ 59.1. For 17-OMe alkylation, the correlation would have been at $\delta_H$ 3.24/$\delta_C$ 56.7. The $^1H$ NMR spectra of authentic samples of **3** and **4** were obtained under identical conditions at the same time, giving chemical shift data that were identical to the published data.[3]

These experiments established that PedO had selectively methylated the terminal 18-OH group of **3** and should therefore have an analogous function in the biosynthesis of pederin (**1**). Interestingly, although **1** carries four methoxy groups, only three *O*-MT genes were identified in the *ped* system.[4] Inspection of the symbiont genome sequence that became recently available (unpublished data) did not indicate the presence of an additional MT gene candidate. The data therefore either suggest that PedA or PedE has a dual function or that the fourth methylation is performed by the beetle host.

This is to our knowledge the first reported biochemical evidence that animals obtain defensive polyketides from bacterial endosymbionts. Uncultivated bacteria have long been proposed to be the true producers of complex polyketides in a wide range of invertebrates.[8] This also includes the bryostatins, for which a convincing PKS candidate has recently been reported from a bryozoan-associated symbiont and shown to be correctly loaded with malonyl building blocks.[9] For cyclic peptides present in tunicates, a bacterial origin has been demonstrated by heterologous gene expression.[10] Our results have interesting ecological and pharmacological implications, an example being the generation of sustainable sources of bioactive polyketides by bacterial production systems. Using the sponge-derived compound **3** as a substrate for PedO, we were able to regiospecifically generate a hybrid polyketide with increased cytotoxicity that has not yet been reported from nature. The available amounts of **3** prohibited chemoenzymatic synthesis on a larger scale. However, the data suggest that coexpression of genes from different pederin-type pathways could provide sustainable sources of nonnatural analogues with interesting pharmacological properties.

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**Supporting Information Available:** Experimental section, LC-MS analysis of methyltransferase assays, and NMR spectra of the PedO product. This material is available free of charge via the Internet at http://pubs.acs.org.

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